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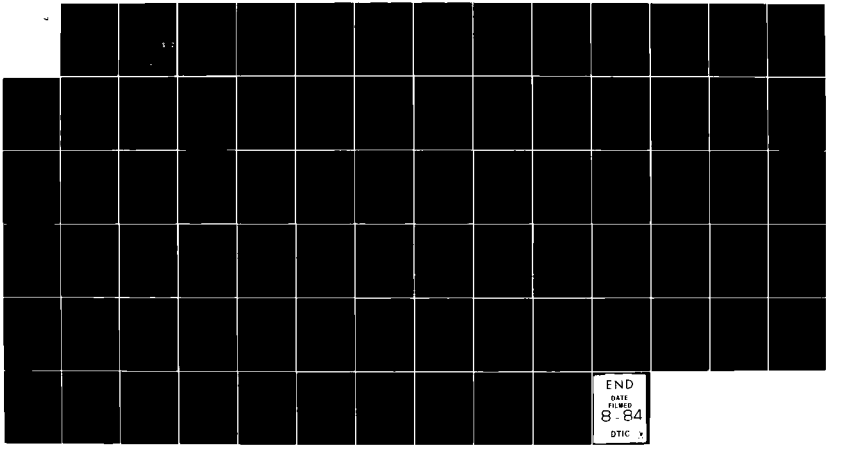

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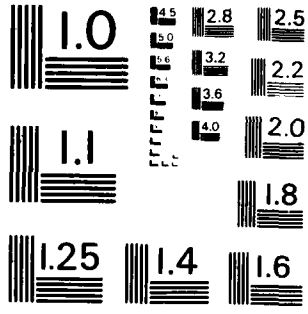

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Revised Final Report

CHEMICAL AND MOLECULAR BIOLOGICAL ASPECTS OF ALKYLHYDRAZINE-INDUCED CARCINOGENESIS IN HUMAN CELLS IN VITRO

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The syntheses for 1,1-(methyl-^{14}C)-dimethylhydrazine, 1,2-(methyl-^{14}C)-dimethylhydrazine, and the important metabolite of 1,2-dimethylhydrazine, namely, ^{14}C-methylazoxymethanol as an acetate derivative are described. Materials of high specific activity were employed to (1) Investigate early events in carcinogenesis; (2) Study DNA modification and damage; and (3) Probe effects of anticarcinogen benzamide, on molecular perturbation of DNA by methylazoxymethanol acetate.

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Final Scientific Report

Chemical and Molecular Biological Aspects of Alkyl-hydrazine-Induced Carcinogenesis in Human Cells in Vitro

By

Donald T. Witiak

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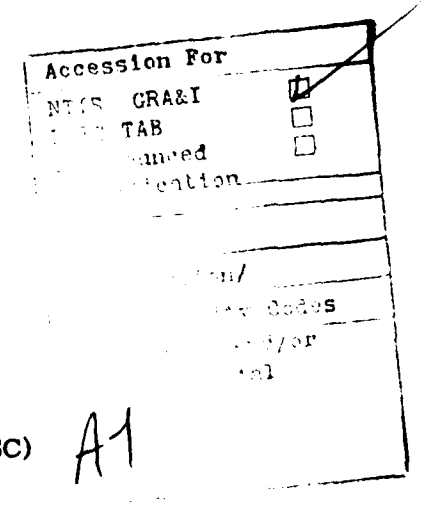

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Attention: Christopher T. Lind, Lt. Col., USAF
Program Manager
Life Science Directorate

Index

Abstract

We have investigated the cytotoxicity, transformation efficiency, methylation of DNA and alkaline labile damage caused by monomethylhydrazine (MMH), 1,1-dimethylhydrazine (1,1-DMH), 1,2-dimethylhydrazine (1,2-DMH), and the metabolite of 1,2-DMH, namely, methylazoxymethanol (MAM) as the acetate (MAMA) on human neonatal foreskin fibroblast (HNF) cells. To accomplish these biological goals, samples of radio labelled compounds of high specific activity were required and the synthesis are reported in this document. Among the four compounds studied, MAMA was most toxic. The effective cytotoxicity ED_{50} doses were 1.35, 6.83, 6.3, and 0.056 mM for MMH, 1,1-DMH, 1,2-DMH, and MAMA, respectively. Except for MMH, the other three compounds transformed HNF cells at an ED_{25} dose as determined by their anchorage independent growth in soft agar. Under these conditions, MAMA and 1,1-DMH were more potent transformers than 1,2-DMH.

HNF cells presumably activated the hydrazines which subsequently undergo reaction with cellular DNA. Using ,^{14}C.-MMH approximately 50% of the radiolabel was associated with unmodified adenine and guanine. This likely reflects metabolism via the one curbon pool. The majority of the remaining label was found in the apurinic acid fraction. No methylation of purine bases was observed. When randomly proliferating cells were treated with either ,^{14}C.-1,1-DMH or 1,2-DMH at a nontransforming dose of 0.17 mM, low levels of methylation of guanine could be detected at the N^7 and 0^6 positions. However, a major portion of the label was associated with the apurinic acid fraction. When cells synchronized by arginine and glutamine deprivation were treated with ,^{14}C.-1, 1-DMH and 1,2-DMH at a transforming dose of 0.5 mM for four to twelve hours, no significant increase in methylation of adenine or guanine was observed. Again, the majority of ,^{14}C.- label

was associated with the apurinic acid fraction. Treatment of synchronized cells with ,^{14}C.- MAMA at a transforming dose of 0.027 mM resulted in methylation of guanine at the N^7 and 0^6 positions. Although methylation at 0^6 was very low, a time-and dose-dependent increase in both 7-MeGua and 0^6-MeGua was observed.

Both 1,1-DMH and 1,2-DMH, at equitoxic doses (ED_{25} equals approximately 5mM or ED_{50}), induced no detectable alkaline-labile lesions in DNA, whereas MAMA did induce single strand breaks in a dose-dependent manner. The reactivity of MAMA may reflect alkylation by the carbinolanine-like carbon. Such reactivity with DNA could cause damage independent of methylation via diazomethane or diazonium ion metabolites and such damage might be difficult to detect owing to the predictable instability of such adducts defined by $CH_3NO{=}NCH_2$-DNA.

Benzamide, an inhibitor of MAMA- induced transformation had no effectd on methylation of DNA and MAMA- induced single strand breaks. At 1.0 mM concentations, this compound completely inhibited MAMA- induced transformation when added at the G,/S phase of the cell cycle. Benzamide also had no effect on total methylation of DNA or on the formation of 7-MeGua. However, approximately a 40% inhibition in the formation of 0^6-MeGua was observed. Addition of benzamide to the cells at a 1.0 mM concentration prior to and with MAMA had no effect on single strand breaks.

The lack of increase in 0^6-MeGua content with increasing dose of 1,1-DMH and 1,2-DMH suggested that factors other than 0^6-MeGua formation play a significant role in transformation. Single strand breaks also seem not to be important because methylhydrazines did not induce such damage detectable using alkaline sucrose gradient sedimentation. Since benzamide, an inhibitor of transformation, had no effect on 7-MeGua content, it would appear that 7-MeGua has no relevance to transformation. Although a small degree of inhibition of 0^6-MeGua was observed,

transformation was completely inhibited. Any correlation between these events is tenuous at best. We also conclude that formation of single strand breaks in these studies, as assessed by alkaline sucrose gradient sedimentation, do not correlate with initiation of transformation.

Experiments are in progress to determine if 1,1-DMH, 1,2-DMH and MAMA can cause an aberrant endogenous methylation of cytosine. Following deamination, such methylation could yield thymine, thereby creating a permanent miscoding of the genetic material.

Summary of Work Accomplished

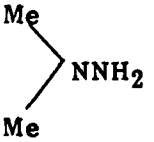
The work accomplished during the tenure of our contract F49620-80-C-0086, 1 July 80 through 30 September 83, is divided into five chapters. Biological results summarized in the abstract to this report could only be accomplished following synthesis of ,^{14}C.-radio labelled 1,1-DMH, 1,2-DMH and MAMA.

Chapter I

Synthesis of 1, 1-DMH (N,N-[Methyl-^{14}C]-dimethylhydrazine) of High Specific Activity

INTRODUCTION

More than forty hydrazines and their derivatives have been found to be carcinogenic to laboaratory animals.[1] This is of considerable concern owing to the high degree of human exposure to such agents.[2] Hydrazines have natural and synthetic sources[2] and the simple hydrazines including N,N-dimethylhydrazine are used as high-energy storage propellants by the U.S. Air Force.[3] Notably, N,N-dimethylhydrazine has been shown to transform human fibroblast cells in culture as assessed by anchorage-independent growth in soft agar and tumor production in athymic nude mice.[4] To further explore events leading to human cell transformation, which may involve macromolecular alkylation and subsequent DNA damage and repair, a facile synthesis for ^{14}C-labeled N,N-dimethylhydrazine was desired. Metholology based on the novel chemistry published at Kaiser et al.[5] is described in this chapter.



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RESULTS AND DISCUSSION

Synthetic intermediate hydrazone 3 was prepared from the corresponding N,N-dimethylhydrazone 2 by exchange with anhydrous hydrazine according to the method of Newkome and Fishel.[6] For radiolabelling purposes, it is critical that hydrazone 3 be free of residual dimethylhydrazone 2 since contamination with unlabelled 2 would lead to lower specific activity of intermediate 4 and target 1. The exchange method, rather than direct reaction of acetophenone with hydrazine, was preferred since the latter method leads to tars and most importantly unwanted azines from rearrangement of acetophenone hydrazone.[6] Fractional distillation and monitoring by NMR analysis of 2.59 ($-NMe_2$ for 2) served to ensure the purity of 3. Synthesis of 4 from 3 was based upon dialkylation of hydrazone N,N-dianions with alkyl halides.[5] Thus, hydrazone 3 was converted to its blue-green dianion using a slight excess of potassium in liquid ammonia. The solution color change from deep blue to blue-green served as a reliable indicator of dianion quality. Thus if a brown color persists, alkylation will be unsuccessful. On the other hand, a yellow solution treated with additional potassium rapidly developed the desired persistent blue-green color. Addition of 2.2 equivalents of ^{14}C-methyl iodide converted the blue-green solution to a pale yellow color signifying the quenching of the dianion by alkylation. Liquid ammonia, ether and any unreacted methyl iodide were distilled into a vented trap. The residue was triturated with ether and the yellow solution was removed by pipette from the crystalline potassium iodide. Radiolabelled hydrazone 4 was converted to 1 using ether saturated with gaseous HCl. Thin layer chromatographic analysis of the hydrolysate was carried out according to procuedures developed by Fiala and Weisburger.[7]

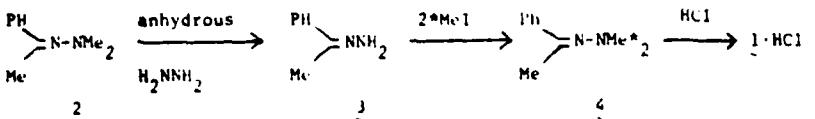

Figure 1: Synthesis of ^{14}C-N,N-Dimethylhydrazine (1)

EXPERIMENTAL SECTION

Nuclear magnetic resonance data were obtained using $CDCl_3$ as a solvent with a Bruker HX-90 spectrometer. Radioactive disintegrations were measured on a Beckman LS-355 liquid scintillation counter using Amersham PCS as a counting cocktail. Thin layer chromatography was carried out on glass plates precoated with Avicel cellulose (250μ thickness), purchased from Analtech. Plates were developed with 2-propanol-water-conc. HCl (130:40:30) and were visualized with Folin-Ciocalteau reagent obtained from Fisher Scientific.

^{14}C-Methyl iodide (58 mCi/mmol) was purchased from Amersham as custom preparation prepared to our specifications by aqueous base extraction and P_2O_5 drying and used without dilution or further purification. Transfer of ^{14}C-methyl iodide was carried out under reduced pressure in a Pope glass vacuum manifold. All glassware were predried under reduced pressure and flushed with ammonia (KOH-dried) for several minutes to obtain an ammonia atmosphere. Ammonia was condensed in an auxiliary flask, dried with Na, and distilled into a reaction flask.

Synthesis of ^{14}C-N,N-Dimethylacetophenone Hydrazone(4). Approximately 12 mg (0.3 mg atom) of potassium was added to about 8 ml of dry liquid ammonia in a 10 ml two necked round bottom flask. To this stirred blue solution was added 20.1 mg (0.15 mmol) of acetophenone hydrazone dissolved in 1 ml of dry ether. The flask was cooled with liquid N_2 and d^{14}C-methyl iodide (20 mCi at 58 mCi/mmol, 0.34 mmol) was added under reduced pressure. The reaction was kept sealed as it was warmed to -78°C (dry ice/acetone). The reaction proceeded as the mixture was allowed to thaw and reach -33°C (liquid NH_3 bp) and was vented through a trap as it exceeded atmospheric pressure. Following venting of the ammonia, ether and residual ^{14}C-methyl iodide, a yellow oil containing white crystals of potassium

iodide was obtained. The oil was dissolved in dry ether (2 ml) and an aliquot taken for counting. The ether solution was found to contain 10.8 mCi (this is a crude radiochemical yield of 54%). The hydrazone 4 was immediately hydrolyzed as decribed below.

N,N-,methyl-^{14}C.-dimethylhydrazine Hydrochloride (1). To the dry ether solution (2 ml) containing hydrazone 4 was added 2 ml of ether saturated with gaseous HCl. The reaction mixture was allowed to stand overnight at room temperature and was centrifuged to pack the white crystalline solid. The supernatant was removed, the crystals washed with dry ether, and centrifuged again as above. The ether supernatant was removed and the crystals dried under reduced pressure (0.5 hr., 0.1 mm hg) and overnight in a nitrogen atmosphere desiccated with P_2O_5. The dried sample was shown to be pure by tlc using authentic 1 as standard. The radiochemical purity was determined to be >97% by scraping and counting each zone. Aliquots were weighted in a dry nitrogen atmosphere, dissolved in methanol and counted in PCS. The specific activity was determined to be 106.0 mCi/mmol. However, from elemental analysis of unlabeled samples prepared under identical conditions, we have found that such samples contain about 0.5 mol of water of hydration. When we consider this water of hydration, we calculate the specific activity to be 114.8 mCi/mmol. The chemical yield of this reaction sequence was 10.02 mg (0.09 mmol, 60% based on starting hydrazone 3) and the radiochemical yield was 10.5 mCi (51%). In some experiments, it was necessary to purify 1 using 20x20 cm Avicel plates. The radioactive band corresponding to 1 was scraped and exhaustively extracted with anhydrous methanol·HCl.

We have observed that at this high specific activity 1 is 80% decomposed after standing 3 months in the dark in 0.1N HCl at 4°C. The shelf life of this material can be lengthened by storing it as the solid hydrochloride salt, desiccated, in the dark at

-70°C. Under these conditions we have found no measurable decomposition (by tlc) over a period of 6 months.

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Chapter II

Synthesis of 1,2-DMH (N,N^1-[Methyl-^{14}C] - Dimethylhydrazine of High Specific Activity

INTRODUCTION

A number of alkyl hydrazines are known to be carcinogenic in small animals.[1] Their extensive use as high energy propellant fuels and their presence in the environment[2] provided the impetus for this research effort. In order to investigate their interaction with human cells, particularly in connection with their transformation potential in tissue culture,[3] radio-labeled methylhydrazines were required to assess metabolic pathways and interaction with critical macromolecules.

Chapter I[4] describes a new synthesis of N,N-[methyl-^{14}Cl] - dimethylhydrazine hydrochloride (UDMH·HCl,1) of high specific activity. This compound was employed in preliminary DNA binding studies using cultured human fibroblast cells.[5] For comparative binding and transformation studies the radiolabeled regioisomer (2, DMH·2HCl) was desired. Methodology utilized for the preparation of [^{14}C] -DMH·2HCl is described and provides a specific activity of 112.5 mCi/mmol. The reaction sequence is shorter (two steps) and affords product of considerably higher specific activity than the route reported by Horisberger and Matsumoto.[6]

$(CH_3)_2NNH\cdot HCl$ $\quad$ $CH_3NHNHCH_3 \cdot 2HCl$

1 $\quad$ 2

RESULTS AND DISCUSSION

Whereas ethyl hydrazinedicarboxylate[7] (3) may be bismethylated by reaction with dimethyl sulfate in 10% NaOH solution[8], the unavailability of high specific activity dimethyl sulfate precluded its usefulness in our studies. Rather, 3 (21.3 mg; 0.166 mmol) was dissolved in 5 ml of absolute ethanol and freshly cut potassium metal (15 mg; 0.334 mg atom) was added. The solvent was removed on a rotavaporator and the residue dried under reduced pressure and dissolved in THF freshly distilled from sodium. ^{14}C-Methyl iodide (2.07 equivalents; 20mCi; approximately 58 mCi/mmol) was transferred to the reaction mixture in a vacuum manifold. Following stirring overnight at room temperature, filtration and solvent removal, a light yellow oil (99.7%) of ethyl N,N'-[methyl-^{14}C]-dimethylhydrazinedicarboxylate (4) was obtained having identical spectral and physical properties with unlabeled compound prepared under analogous conditions. No monomethylated compound was present in the mixture as determined by NMR analysis. Interestingly, N,N'-dibenzoylhydrazine, under these conditions, afforded only monomethylated product and these results are in agreement with Horisberger and Matsumoto.[6] Refluxing 4 in excess concentrated HCl solution for 72 hrs. followed by solvent removal under reduced pressure (rotavaporator) yielded 2 which was purified using Avicel cellulose thin layer chromatographic plates (14.6 mg; 64% from 3; 112.5 mCi/mmol) and shown to be chromatographically identical to authentic unlabeled DMH·2HCl.

$EtO_2CNHNHCO_2Et$ $EtO_2CN(\overset{*}{C}H_3)N(\overset{*}{C}H_3)CO_2Et$

3 4

EXPERIMENTAL SECTION

Nuclear magnetic resonance data were obtained in $CDCl_3$ using a Brucker HX-90 spectrometer. Radioactive disintegrations were measured on a Beckman LS-355 liquid scintillation counter using Amersham PCS as a counting cocktail. Thin layer chromatography was carried out on glass plates precoated with Avicel cellulose (250μ thickness) purchased from Analtech. Plates were developed in 2-propanol:conc. HCl: water (150: 35:15) and were visualized with Folin Ciocalteau reagent obtained from Fisher Scientific.

^{14}C-Methyl iodide (58 mCi/mmol) was purchased from Amersham prepared to our specifications by aqueous base extraction and P_2O_5 drying and used without further purification. Transfer of ^{14}C-methyl iodide was carried out under reduced pressure in a Pope glass vacuum manifold. All glassware were predried under reduced pressure.

The specific activity of 2 was determined by drying and weighing an aliquot in a desicated balance. The weighted material was dissolved in one ml of H_2O and 10 ml of PCS (Amersham) was added. Counting efficiency was determined using Oxi-Test standards from the Radiomatic Instrument and Chemical Company. The ,^{14}C.-DMH·2HCl was found to be stable over 5 months when stored as a solid at -70°C. Appreciable chemical decomposition was observed following 1 month at -20°C. Ethyl hydrazinedicarboxylate (3) was synthesized according to the method of Rabjohn.[7]

Ethyl N,N'-[Methyl-^{14}C]-dimethylhydrazinedicarboxylate (4): Under anhydrous conditions, absolute ethanol (5 ml) was added to 29.3 mg (0.166 mmol) of ethyl hydrazinedicarboxylate (3) in a 10 ml pear shaped flask. To this suspension was added 15 mg (0.384 mg atom) of freshly cut potassium metal; on shaking, a clear solution was obtained. Excess ethanol was removed under reduced pressure

(rotavaporator) at room temperature. The residue was dried under reduced pressure (0.1 mm; 65°C; 4 hr) and suspended in 5 ml of THF freshly distilled from sodium. The suspension was frozen in liq. N_2 and [^{14}C]-CH_3I (0.344 mmol; 20 mCi; 58 mCi/mmol) was transferred to the reaction flask under reduced pressure. The reaction flask was maintained under liq. N_2 for 1 hr, allowed to warm to room temperature and stirred overnight (approx. 18 hr.). The reaction mixture was filtered (scintered glass funnel) and the residue washed thoroughly with dry THF. The filtrate and washings were combined. Following solvent removal at room temperature and under reduced pressure, a residual light yellow oil was obtained. This oil was durther dried under reduced pressure (rotavaporator, 40°C; 15 min.) affording 34.5 mg (99.7% by weight) of radiolabeled 4, ^{1}H-NMR ($CDCl_3$) 1.24 (t, 6H, J=7.5 Hz, 2 -O-C<u>H</u>$_2$-CH_3), 3.09 (S,6H-NCH_3NCH_3-), 4.16 (a,4H, J=7.5 Hz, 2-O-CH_2-CH_3).

<u>N,N'-[Methyl-^{14}C]-dimethylhydrazine Dihydrochloride (2)</u>: Ethyl N,N'-[methyl-^{14}C]-dimethylhydrazinedicarboxylate (4) (34.5 mg; 0.166 mmol) was dissolved in 4 ml of conc. HCl solution and refluxed with stirring for 72 hr (oil bath remp. 150°C). The reaction mixture was cooled (room temperature), the condenser rinsed with conc. HCl and the combined HCl solutions concentrated under reduced pressure (40°C). The residue was drid utilizing several additions of methanol followed by distillation under reduced pressure (rotavaportator) affording 20.6 mg of white residue (MeOH soluble). The residue was dissolved in a minimum volume of methanol and applied to one 20 x 20 cm Avicel cellulose plate and developed in 2-propanol:conc. HCl: water (150: 35:15). The radioactive band corresponding to DMH·2HCl was scraped and eluted with 30 ml of 50% HCl (v/v). Solvent removal of the eluate under reduced pressure and azeotropic drying with methanol afforded 14.6 mg (64% from 3) of $CH_3NHNHCH_3$·2HCl·$O.5H_2O$ (112.5 mCi/mmol). Water of hydration was independently determined by elemental analysis of unlabeled material prepared under identical conditions.

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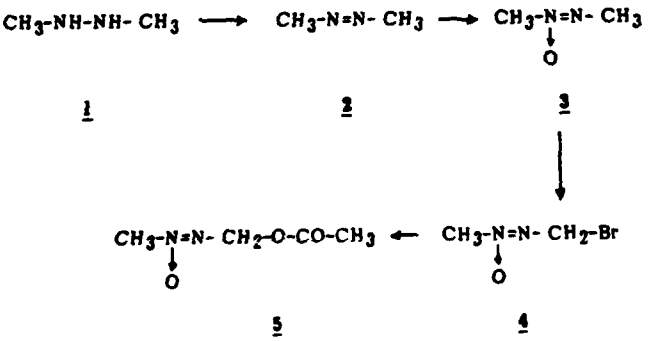
Chapter III

Synthesis of ^{14}C.-labelled Methylazoxymethanol Acetate (MAMA) of High Specific Activity

Cycasin (β-C-glucosyl-azoxymethane)[1,2] is hepatotoxic and carcinogenic in rats[3,4] and this toxicity is attributable to the aglycone, methylazoxymethanol (MAM)[5,6], which is also a metabolite of 1,2-dimethylhydrazine. MAM-acetate has increased stability, reduced volatility, retains toxicity[7,8] and is a very potent transformer of human fibroblasts in culture (manuscripts in preparation). Although the synthesis of ^{14}C-MAM-acetate was reported by Horisberger and Matsumoto[9], their synthesis of this compound, stadrting with commercially prepared ^{14}C-labelled azomethane, yielded only small quantities of labelled product. It was suggested that poor yields may reflect autoradiolysis. In contrast, in this chapter we report the micro-scale synthesis of ^{14}C-MAM-acetate of near theoretical specific activity and in significantly higher yields.

DISCUSSION

Using a modification of the synthetic scheme reported by Horisberger and Matsumoto[9] the synthesis of ^{14}C-MAM-acetate (5) was accomplished by oxidation of N,N'-,methyl-^{14}C.-dimethylhydrazine (1) to ^{14}C-azomethane (2) and subsequently ^{14}C-azoxymethane (3). Allylic type bromination followed by reaction with silver acetate afforded 5.



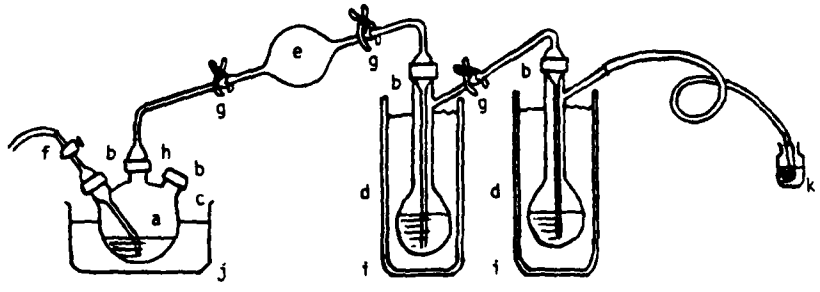
Although reaction conditions using cold material were optimized at each step, the reaction conditions for the radiolabelled material required considerable modification. Micro-scale production of high spoecific activity 5 was carried out using the described apparatus. The 25 ml reaction flask and 10 ml traps were critical; poor yields of 3 were obtained with unlabelled material on the micro-scale when these vessels were 50 and 25 ml, respectively.

Under these conditions 1 was converted to 3 in 80% yield; a significant improvement over the 31% average reported by Horisberger and Matsumoto[9]. The argon flow rate of 5-6 ml/min is critical since a slower rate does not adequately carry the azomethane from the reaction flask and at faster flow rates azomethane escapes from the traps. Both traps were charged with the less polar methylene chloride solvent substituted for previously used ether[9]. Thus, the m-chloroperbenzoic acid is retained on the column during chromatographic purification on basic alumina.

The desirable temperature of trap #1 is 10 - 12°C. Too low a temperature results in precipitation of m-chloroperbenzoic acid and this in turn decreases conversion of azomethane (2) to azoxymethane (3). Gas flow and evolution of radioactive gas was routinely monitored by bubbling the trap effluent through scintillation cocktail. During 20 - 30 mCi reactions only 3 - 10 μCi of ^{14}C could be detected in the scintillation vial. A significant difference in reaction time was required for bromination using labelled vs unlabelled azoxymethane. For unlabelled material bromination was complete in 2 - 3 hr, whereas azoxymethane of high specific activity always required a 5 - 6 hr reaction time (4 experiments). Although it is tempting to propose an isotope effect to explain these results, further work is necessasry to substantiate such a possibility.

Miligram quantities of MAM-acetate (5) were purified by thin layer chromatography (TLC) and visualized under UV lilght. The MAM-acetate band was scraped and eluted with methylene chloride. Caution should be exercised during solvent evaporation so as not to lose product. TLC-purified ^{14}C-MAM-acetate was analyzed by HPLC under conditions markedly different than those reported by Fiala et al.[11] Neither HPLC metholdologies may be utilized for preparative purification owing to difficult product recovery from the eluting solvent (approx. 12% $MeOH/H_2O$). Long range spin coupling (J=1.46) between $N-CH_3$ and NCH_2 functions confirmed the structural assignment.

Assembly for the Micro-scale Preparation of Azoxymethane



a. 25 ml three necked flask. b. 14/20 "Thread-Tite" joint. c. "Thread-Tite" teflon/silicon septum cap. d. 10 ml bulb long necked trap with side arm and including a gas bubbler that extends close to the bottom of trap. e. 10 ml bulb filled with anhydrous $CaCl_2$. f. Gas inlet tube extending close to the bottom of 3 necked flask. g. 12/5 ball and socket joint secured with a clamp. h. gas outletd tube secured to reaction flask. i. dewar flask j. ice bath k. scintillation vial with 10 ml cocktail and a pipet bubbler.

EXPERIMENTAL SECTION

NMR data were obtained in 100% $CDCl_3$ using an IBM NR/80 spectrometer. Radioactive disintegrations were measured on a Beckman LS-355 liquid scintillation counter using Amersham PCS or NEN formula 963 as a counting cocktail and dpm were determined using OXI-TEST internal standard from Radiomatic Instrument and Chemical Co. Inc. Standard N,N'-dimethylhydrazine dihydrochloride was purchased from Aldrich; m-chloroperbenzoic acid and unlabelled MAM-acetate were purchased from Sigma. TLC plates were silica gel GF, 10 x 20 cm, 250 micron, glass plates purchased from Analtech. HPLC was carried out using a Laboratory Data Control (L.D.C.) Gradient System controlled by a Commodore PCM 1611 control module. The column effluent was monitored using a L.D.C. Spectromonitor III variable wavelength UV detector and the radioactivity was measured by radioactive flow detector FLO-ONE model HP using Flo-Scint II cocktail purchased from Radiomatic Instrument and Chemical Co. The column was a L.D.C. Excalibar Spherisorb ODS 5μ, 4.6 x 250 mm. All glassware utilized had "Thread-Tite" 14/20 joints and caps purchased from Reliance Glass Work, Inc.

N,N'[-Methyl-^{14}C]-dimethylhydrazine dihydrochloride (1):

Dimethylhydrazine (1), having a specific activity of 112.5 mCi/mmol, was prepared in 50% yield by the method of Kumar et al.[10] The purity of the compound was determined by comparing its TLC with that of standard N,N'-dimethylhydrazine dihydrochloride.

^{14}C-Azomethane (2) and ^{14}C-Azoxymethane (3):

These compounds were prepared in sequence without separating 2. Thus, in a 25 ml 3-necked round bottom flask fitted with a teflon/silicon rubber septum and a gas inlet tube, was placed 223 mg of Amberlite IRA-93 previously washed 4-5 times

with water. Yellow mercuric oxide 56 mg, (0.26 mM) and 1 ml of water was added. Two cooled traps each containing 10 ml of methylene chloride were utilized. To trap #1 was added 50 mg of m-chloroperbenzoic acid. Trap #1 was cooled to approx. 10°C with cold water and trap #2 was cooled in an ice-water bath (approx. 0°C). The reaction flask was cooled in an ice bath and 24.36 mg, (0.178 mM, 20 mCi, 112.5 mCi/mmol) of N,N'-[methyl-^{14}C]-dimethylhydrazine dihydrochloride (1) dissolved in 1 ml of water was added by syringe through the septum. The ice bath was removed after 30 minutes and the reaction mixture was stirred at room temperature for 2 hours. Argon was then bubbled through the reaction mixture at a flow rate of 5-6 ml/min and the temperature of the oil bath was gradually raised from room temperature to 80-85°C. The reaction was stirred for an additional 2-3 hours at this temperature. Heating and gas flow were stopped and the solutions from the two traps were combined and stored at -4°C for 24 hrs. The solution was brought to room temperature and passed over basic alumina (2 gm) using a 1 x 15 cm column. The column was washed with methylene chloride (5 ml) and the eluent was distilled at 70°C using a 15 cm vacuum jacketed vigreaux column. The total radioactivity in the residue was 16 mCi. The yield of ^{14}C-azoxymethane (3) was 80% based on 1 (NMR of the residue from unlabelled 3 exhibited NMR ($CDCl_3$) 3.18(bs, 3H,=NCH_3), 4.05 (bs, 3H, CH_3-NO=).

^{14}C-Bromoazoxymethane (4) was prepared by placing 16 mCi (0.145 mM) of 3, 70 mg (0.39 mM) of N-Bromosuccinimide and 3 ml of carbon tetrachloride in a 10 ml pear-shaped flask fitted with a water condenser protected by a calcium chloride tube. The mixture was stirred at 50-55°C under a 60 watt lamp held at a distance of 2 cm. Development of a light orange color in the mixture indicated reaction completion (5-6 hrs.). The mixture was cooled to room temperature and filtered through a

disposable pasteur pipet plugged with glass wool. The filtrate containing 4 was immediately converted to ^{14}C-MAM-acetate without further purification.

^{14}C-Methylazoxymethanol acetate (5): To the solution of 4 in carbon tetrachloride was added 85 mg (0.50 mM) of silver acetate. The mixture was protected from light, stirred at room temperature overnight, and filtered using a pasteur pipet plugged with glass-wool. The solvent was removed at 77°C using a short path distillation head affording a residue containing a 10 mCi ^{14}C. The crude product was purified (TLC) on silica-gel using ethyl acetate:hexane (1:2) as eluting solvent. The band which chromatographed with unlabelled standard 5 was scraped, eluted with methylene chloride, and evaporated on a rotary evaporator at room temperature (caution must be exercised to avoid loss of 5) to afford 4.03 mCi (20.1% yield) of pure 5 exhibiting a specific activity of 110.3 mCi/mmol (determined from a weighted aliquot). The chemical and radiochemical purity of TLC-purified 5 was analyzed by HPLC. The only detectable contaminant was 3 (2.8%). TLC purified 5 was eluted using a 5-15% methanol linear gradient at 0.8 ml/min over 67 minutes. Compounds 3 and 5 were detected by monitoring both absorbance at 235 nm (0.05 AUFS) and dpm at 85% static efficiency using a 3:1 cocktail to eluent ratio and a 0.5 ml flow cell. Retention times were 19.4 and 33 min. for 3 and 5, respectively. NMR ($CDCl_3$) for both labelled and unlabelled standard exhibited δ 2.15 (s, 3H, CH_3CO), 4.06 (t, 3H, J = 1.46 Hz, CH_3NO) 5.37 (q, 2H, J = 1.46 Hz, = NCH_2O). The distillate from crude 5 contained 6 mCi of 3. This distillate on rebromination, acetylation and purification by TLC afforded 900 μkLCi of pure 5. The total yield of 5 was 4.93 mCi (24%) based on 1.

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Chapter IV

DNA Modification and Damage by Dimethylhydrazine and Methylazoxymethanol Acetate.

INTRODUCTION

Alkylhydrazines 1,1-dimethylhydrazine (1,1-DMH) and 1,2-dimethylhydrazine (1,2-DMH) and the metabolite methylazoxymethanol acetate (MAMA) have been shown to induce cancer in vivo in several species of rodents producing a variety of neoplasms (10,14,15,18,21,34,36,39,43-46,48) with the distribution of tumors depending upon the dosage, schedule and route of administration. In the past decade, the more potent animal carcinogen 1,2-DMH has received much attention as an organotrophic carcinogen because of its ability to induce colon cancer in rats (10,34). The mechanism by which 1,2-DMH exerts its carcinogenic action has been postulated to be similar to that of other simple alkylating carcinogens in that the metabolism of the procarcinogen leads to the formation of an active alkylating agent (methyl diazonium ion) within susceptible tissue and subsequently alkylates a variety of macromolecules like nucleic acids and proteins (8,16,17).

Carcinogenecity of alkylating agents has been attributed to their ability to alkylate DNA at specific sites in the DNA. Alkylation of oxygen atoms of purines and pyrimidines of DNA has been suggested to be critical to mutagenesis and carcinogenesis (40) and a positive correlation has been found between tumorigenesis and the formation and persistence of the promutagenic lesion O^6 alkylguanine in target tissues (12,23-25,31,33). Other studies have demostrated little correlation between persistence of O^6 alkylguanine and susceptibility to carcinogenesis (27,28).

Relatively little information is known concerning the carcinogenic effect of methyl hydrazines on human cells. Autrup and co-workers (3,4,13) have reported that human colon and bronchus maintained as an explant culture metabolized 1,2-DMH resulting in methylation of DNA and protein. In human colon explant culture, Autrup et al. (2) have demostrated that the main alkylation of the DNA occurs at O^6 and N^7 positions of guanine with an O^6 to N^7 alkylation ratio of 0.12. However, these investigators did not measure a biological response to the alkylating agents. We have previously reported that 1,1-DMH and MAMA can induce neoplastic transformation in vitro while 1,2-DMH did not (27,28).

Using these three related alkylating agents which differ in their oncogenic potency, we describe the relationships between DNA damage, DNA alkylation, cytotoxicity, and transformation in human cells.

MATERIALS AND METHODS

Chemicals, Radiochemicals and Cell Culture Media

1,2-DMH, MAMA, ribonuclease A and pronase were obtained from Sigma Chemicals Co., (St. Louis, MO). The 1,1-DMH, obtained from Aldrich Chemical (Milwaukee, WI), was redistilled before use. The purity of these carcinogens was 98-99.5% as determined by thin layer chromatography and NMR. Radiochemicals, [2-^{14}C]-thymidine (S.A. 54.6 mCi/mmole) and [methyl-^{3}H]-thymidine (S.A. 20 Ci/mmole) were supplied by New England Nuclear Corporation, (Boston, MA). [^{14}C]-1,1-DMH (S.A. 110-115 mCi/mmole), [^{14}C]-1,2-DMH (S.A. 110-115 mCi/mmole) and [^{14}C]-MAMA (S.A. 110-112 mCi/mmole) were synthesized in the radiochemistry laboratory at The Ohio State University Comprehensive Cancer Center. Powdered cell culture media and supplements were purchased from Grand Island Biological Company, (Grand Island, NY) and MA Bioproducts, (Walkersville, MD), respectively.

Cell Culture

Primary cultures of human neonatal foreskin fibroblasts (HNF) were obtained from routine circumcision tissue dispersed with collagenase (35). These cell populations at low population doublings (PDL) were serially subpassaged in complete medium (CM) composed of minimum essential medium (MEM), containing 25 mM Hepes at pH 7.2 supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2.0 mM glutamine, 50 μg/ml gentocin, 0.2 % sodium bicarbonate and 10% fetal bovine serum, in a 4% CO_2 enriched air atmosphere.

Chemical Cytotoxicity

Cytotoxicity was assayed by the method described earlier (32). Briefly, cultures of 40 cells/cm^2 in 25 cm^2 dishes were treated with the carcinogens for 24 hrs at different concentrations. After 7-10 days, the cultures were fixed with 3% formalin in PBS, stained with hematoxylin and the colonies of more than 50-100 cells were counted. These concentrations of the compounds when used in transformation experiments at 10^4 cells per cm^2 exhibited no altered cytotoxicity.

Carcinogen Treatment and Transformation

Exponentially growing preconfluent HNF cells at PDL 3-5 were blocked at G_1 phase of the cell cycle by seeding the cells in Dulbecco's modified Eagle's minimum essential medium, deficient in arginine and glutamine, supplemented with 1.0 mM sodium pyruvate, 50 μg/ml gentocin and 10% dialyzed FBS (dialyzed against Dulbecco's E. MEM lacking arginine and glutamine) (28-30). Twenty-four hrs later, the cells were released from the block by feeding with CM supplemented with 10% FBS and 0.5 U/ml insulin. Ten hrs after release, when cells were entering early S phase, the carcinogen(s) 1,1-DMH, 1,2-DMH and MAMA were added. The carcinogen stock solutions were freshly prepared in CM with no serum just before use. At the end of the 12 hr treatment period, control and treated cell cultures were rinsed three times with CM minus serum and subpassaged at a 1:2 split ratio into CM containing 0.8 mM nonessential amino acids, 2X vitamins and 20% FBS (selection medium). When the cell populations were 75% confluent, they were serially passaged at a 1:10 split ratio into the selection medium. After 16-18 PDL, 50,000 cells from rapidly proliferating nonconfluent culture were seeded into 2.0 ml of a 0.35 % agar in modified Dulbecco's LoCal medium over a 5 ml of 2% agar base made

in RPMI 1629 growth medium (28,29). The soft agar cultures were incubated at 37°C in a 4% CO_2 enriched air atmosphere. The cultures were fed once in seven days with approximately 0.2 ml Dulbecco's LoCal medium supplemented with 20% FBS. Three weeks later, the plates were scored for colony formation.

Incubation with Carcinogen and Isolation of DNA

At the onset of scheduled DNA sythesis, i.e., ten hrs after release from the block, radiolabeled carcinogens [^{14}C]-DMH (0.027 mM, 0.17 mM and 0.5 mM; 37.5, 12 and 15 mCi/mmole, respectively), [^{14}C]-1,2-DMH (0.17 and 0.5 mM; 12 and 7.2 mCi/mmole, respectively) and [^{14}C]-MAMA (0.0175, 0.02 and 0.03 mM, 55 and 110 mCi/mmole, respectively) were added to the cells in early S, and incubated for 6 hrs unless otherwise specified at 37°C. At the end of the treatment period the plates (a total of 1-1.5 x 10^8 cells) were rinsed 2X with MEM; the cells were scraped from the culture dish, and washed three times with MEM by centrifugation. The cell pellet was finally suspended in SSC buffer (pH 7.0) at 10^7 cells/ml and lysed with 1% SDS. The cell lysate was extracted with an equal volume of redistilled phenol reagent (phenol 500 g, m-cresol 70 ml, 8-hydroxy quinoline 0.5 g, and 2X distilled water 55 ml), for 15 min. The contents were centrifuged at 10,000 x g for 30 min at room temperature to separate the organic-aqueous layers. The aqueous layer was removed and the phenol layer re-extracted with an equal volume of SSC buffer again. The pooled aqueous layers were re-extracted with phenol reagent once more. To the aqueous layer, 1/10 volume of 2M sodium acetate, at pH 5.0, was added followed by two volumes of ethanol at -19°C to precipitate the DNA. Any contaminating RNA and protein were removed from the DNA by incubating the DNA (1.0 mg/ml) in SSC buffer at 37°C for 30 min with 50 μg/ml of preheated

pancreatic ribonuclease (80-100°C for 10 min) followed by incubation with 100 μg/ml of pronase (predigested at 45°C for 1 hr), for 60 min at 37°C. The DNA was re-extracted again with an equal volume of phenol until there was very little interphase. DNA was finally precipitated with two volumes of 95% ethanol at -19°C in the presence of 0.2 M sodium acetate at pH 5.5-6.0. The precipitated DNA was washed with ethanol (-19°C) followed by ether (-19°C) and dried under a stream of nitrogen or argon.

The purified DNA was hydrolysed to purine bases and pyrimidine oligo nucleotides by heating at 70°C for 30 min in 0.1 M HCl. The sample was centrifuged and the supernatant chromatographed with appropriate standards, (3-methyladenine, 7-methylguanine, O^6-methylguanine, adenine and guanine) on Sephadex G-10 column (1.5 x 80 cm) with 50 mM ammonium formate buffer, pH 6.8, or by cation exchange high pressure liquid chromatography (HPLC). (The standards used for HPLC were N^2-methylguanine, 7-methylguanine, O^6-methylguanine, adenine, 1-methyladenine, 3-methyladenine, 7-methyladenine, N^6-methyladenine and 3-methylcytosine). Chromatographic separation by HPLC was performed using a Laboratory Data Control (LDC) HPLC system with a Partisil-10 SCX M9 column (9.0 x 250 mm) in series with Partisil-10 SCX column (4.6 x 250 mm). The methylated products were eluted at 4.0 ml/min with a buffer which changed from 20 mM ammonium formate in 6% methanol, pH 4.0 to 200 mM ammonium formate, pH 4.0, in 8% methanol over 25 min using a concave gradient. Elution was continuously monitored by absorbance at 254 nm. One ml fractions were collected and assayed for radioactivity on a Beckman LS9000. When separated on Sephadex-G10 column, peak fractions were pooled, reduced in volume by lyophilizing, and radioactivity was measured by counting in Instagel or 963 scintillation cocktail, also using a Beckman LS9000 scintillation counter.

Alkaline Sucrose Gradient Analysis

Randomly proliferating cells were labeled overnight with [methyl-^{3}H] or [2-^{14}C]-thymidine (1.0 or 0.2 μCi/ml respectively), incubated in fresh medium for 2 hrs and then treated with carcinogen for 1,3,12, or 24 hrs. At the end of the treatment period, cells were washed with PBS containing 0.04% EDTA and detached from the dish in 2.0 ml of PBS:EDTA using a rubber policeman. The cells were pelleted by centrifugation at 1000 rpm for 5 min, washed once and resuspended in PBS:EDTA at 5 x 10^{5} cells/ml. The cell suspension containing approximately 5 x 10^{4} cells was carefully layered on top on a 0.3 ml of lysis solution (1 M NaOH: 10 mM EDTA) layered on top of a 4.5 ml, 5-20% w/v sucrose gradient containing 2 M NaCl, 0.33 M NaOH and 0.01 M EDTA and lysed in the dark for 3 hrs as described previously (42). The gradients were centrifuged in an S.W. 50.1 rotor for 110 or 145 min at 25K in a Beckman L8-55 ultracentrifuge. Approximately 25 fractions (0.2 ml each) were collected from the gradient and the radioactivity counted using 5.0 ml of Instagel scintillation cocktail in a Beckman LS9000 scintillation counter. The gradients were precalibrated for molecular weights using the following phage DNA markers: lambda, T2 and T7.

The number of alkaline labile sites (breaks due to apurinic sites and phosphotriesters) were calculated as: sites per 10^{8} dalton = (1/Mn (treated) - 1/Mn (control)) x 10^{8} where Mn is the number average molecular weight. When synchronized cells were used, exponentially growing cultures were first labeled for 24 hrs and then the cells were synchronized as mentioned earlier. At the onset of scheduled DNA synthesis, the cells were treated with carcinogen(s) for 12 hrs and processed further as indicated for randomly proliferating cells.

RESULTS

Cytotoxicity and Transformation

We have measured the colony formation, (percent-cloning-efficiency), in monolayer culture as a criterion for cytoxicity and colony formation in soft agar, (50 cells or more per colony), for the expression of anchorage independent growth, (transformation). Chart 1 illustrates the cytotoxicity data for the two DMH compounds and the acetylated metabolite, MAMA. As expected from our earlier results (28) MAMA is found to be much more toxic to cells than either DMH compound. However, in the present study we find 1,1-DMH and 1,2-DMH to be much less toxic than reported earlier (27). The respective ED_{50} toxic doses were 6.3 mM for 1,2-DMH, 6.83 mM for 1,1-DMH and 0.056 mM for MAMA. The cytotoxic-noncytotoxic intercept values, (dy/dx = 0), ED_O, were 5.26 mM for 1,2-DMH, 4.17 mM for 1,1-DMH and 0.009 mM for MAMA (Chart 1). It was observed that all three compounds could transform cells at either a non-toxic dose or toxic dose, ED_{25}, (Table 1). These carcinogen treated cells have been shown to exhibit cell invasiveness in chick embryo skin, human malignancy specific cell-surface antigenic determinants (19), and produce tumors in pre-irradiated nude mice (28). Earlier we reported that 1,2-DMH did not transform human fibroblasts (27) but now we find that cells could be transformed but at a low frequency. The discrepancy in toxicity values presented here compared to those published earlier, (28,27) was not unusual. In each situation the compounds were repurified and the purity of all compounds used in these experiments was $\pm$ 98% as determined by NMR. The lack of congruity of these results compared to previously published results was, to the best of our knowledge, due to different methods of synthesis of the hydrazines. The

results reported on before were based on a purity of 95% as determined by infrared examination of the compounds prior to use. The source of earlier lots of the hydrazine compounds was the Air Force repository at Wright Patterson Air Force Base, (Ms. Marilyn George) while recent lots were obtained from commercial sources.

Binding of Carcinogens to DNA

HNF cells activated all the three carcinogens into metabolites that reacted with total cellular DNA (Table 2). The level of incorporation of label from 1,1-DMH and 1,2-DMH into total cellular DNA was approximately similar in randomly proliferating cells and in synchronized cells, expressed as dpm's per mg of DNA with the following exception. Synchronized cells, treated with 1,2-DMH in the early S phase incorporated 2.0 fold more radiolabel into cellular DNA compared to similarly treated randomly proliferating cells. It is interesting to note that although the level of incorporation of label into DNA was high when cells were treated with 1,2-DMH, it was a poor transforming agent.

Digestion of the total treated cellular DNA and subsequent separation of the modified bases by HPLC yielded the following results. The individual fractions, as they eluted from the column, were identified by co-chromatographed markers, (Chart 2). When randomly proliferating cells were treated with either 1,1-DMH or 1,2-DMH at a nontransforming dose of 0.17 mM, for 24 hrs, 60-76% of the label was associated with the apurinic acid fraction of the digested DNA, (Table 3). Approximately 0.23% and 0.09% of the total label of 1,1-DMH and 0.17% and 0.07% of the total incorporated label of 1,2-DMH into DNA was associated with N^7 - methylguanine and O^6-methylguanine, respectively, (Table 3). Treatment of the cells at the onset of S phase of the cell cycle for a period of 6 hrs at a non-toxic

transforming dose of 0.5 mM for either 1,1-DMH or 1,2-DMH resulted in 90-91% of total incorporation of the label into the apurinic acid fraction, 0.24% and 0.20% into the N^7 methylguanine fraction and 0.08% and 0.01% into the O^6methylguanine fraction of 1,1-DMH and 1,2-DMH, respectively (Table 4). It was interesting to note that in 1,1-DMH treated cells the percentage of methylation of guanine at the N^7 position and O^6 position was similar in randomly proliferating as well as synchronized cells when the cells were treated at a non-tranforming and a transforming non-cytotoxic dose. However,when the synchronized cells were treated with a transforming dose of 1,2-DMH we observed approximately a sevenfold decrease in the specific methylation of the O^6 position in guanine. The peaks of modified bases of the cells treated at 0.5 mM at a transforming dose of 1,1-DMH are presented in Chart 2. Moreover, treatment of cells with 1,1-DMH at the non-cytotoxic transforming dose of 0.5 mM for 12 hrs instead of 6 hrs also did not increase the methylation of O^6-guanine. Treatment of synchronized cells with 0.03mM MAMA, a cytotoxic transforming dose, (Table 1, 4), resulted in quantitatively more incorporation of the radiolabel into the N^7 position of guanine, (0.45% of total). This modification of the N^7 position of guanine was similar to that observed for 1,1-DMH treatments but tenfold higher than 1,2-DMH treatments. Furthermore, extension of treatment times with MAMA from 3 to 6 hrs resulted in a two fold increase in the amounts of the N^7-methylguanine and O^6-methylguanine, however, the ratios of modification remained similar, 0.18 to 0.26, (Table 5).

These results presented here in this report differ from those reported by others for hydrazine and/or metabolite treatment in human cell systems. For example, in cultured human bronchus explants treated with 1,2-DMH, Harris et al. (12), have reported that 51% of total radioactivity was incorporated into the O^6-methylguanine and 16% into N^7 methylguanine. Autrup et al. (2), using cultured

colon explants, have demonstrated that 23% of the radiolabel was incorporated into O^6-methylguanine and 46% into N^7-methylguanine. Although these experimental DNA binding results reported on here differed from those reported on by Harris et al. (12) and Autrup et al. (2-4), their data was compiled from explant cultures treated with hydrazines, whereas we carried out our experiments under conditions to evaluate both the cytotoxic and carcinogenic response of 1,1-and 1,2-DMH treated cells; this was not the case with Harris et al. (13) and Autrup et al., (2-4).

Carcinogen Treatment and Sedimentation Analysis

The binding data presented here illustrates that a major amount of radiolabel was associated with the apurinic acid fraction. This could be due to: a) formation of phosphotriesters, b) methylation of pyrimidines, and c) de novo synthesis of pyrimidines. If incorporation of radiolabel into the apurinic acid fracion was due to phosphotriester formation, then we should have observed single strand break following treatment of the cells with 1,1-DMH and 1,2-DMH. No detectable single strand breaks were observed in both nontoxic and toxic transforming doses of 1,1- and 1,2-DMH. With MAMA, however, a dose dependent increase in strand break was observed (Chart 3). Low levels of break (0.27 and 0.67 breaks per 10^8 daltons at a dose of 30 and 75 μM MAMA) were observed. These observations suggested that the breaks may be due to the presence of depuriniation sites rather than due to the formation of phosphotriesters in the treated DNA.

DISCUSSION

Since the suggestion by Goth and Rajewsky that sensitivity of young rat brain to carcinogenecity by ethylnitrosourea may result from a longer persistence of O^6 ethylguanine adducts in brain, compared to other organs, many investigations of organ specificity of carcinogenesis have demonstrated that target organs have less ability to remove O^6 alkylguanine lesions than do nontarget organs (6,22). However, studies with 1,2-DMH in rats and hydrazine in hamsters have also demonstrated that accumulation of O^6-methylguanine occurs to a greater extent in liver than in any other organs and its persistence did not correlate with the hepatocarcinogenecity (9,37) indicating that factors other than the formation and persistence of O^6 alkylguanine other promutagenic lesions such as O^2-alkylthymine, O^4-alkylthymine, O^2-alkylcytosine (40), 3-methylcytosine (41) must be considered.

We have shown that HNF cells are capable of metabolizing 1,1-DMH, 1,2-DMH and MAMA and alkylating the cellular DNA. However, the degree of DNA modification was less than that reported by Harris et al.(13) in human bronchus explants and Autrup et al. (2-4) in human colon explants. To our knowledge, methylation of HNF cells by 1,1-DMH , 1,2-DMH and MAMA has not been reported previously. Harris et al. (13) using human bronchus have shown that treatment of explants for 24 hrs with a noncytotoxic dose of 1,2-DMH (1.29 mM) the DNA was methylated. The level of total binding ranged from 361-1459 pmoles/mg DNA and 51% of the total counts were associated with O^6-methylguanine and 16% with N^7-methylguanine. The ratio of O^6/N^7 guanine base was 3.18. Autrup et al. (3) treated human colon explants with 1,2-DMH (100 μM) for 24 hrs and observed the total level of binding to be 62 $\pm$ 64 pmoles/mg DNA. About 23% of the total counts

were associated with O^6-methylguanine and 46% was associated with N^7-methylguanine. The ratio of O^6/N^7 was 0.5. In the present study we have found after the treatment of randomly proliferating HNF cells for 24 hrs with a nontransforming dose of 1,1-DMH and 1,2-DMH (0.17 mM), the total binding radiolabel was 9 and 11 nmoles/mg DNA respectively, which are much higher than those reported for bronchus and colon explants. Both 1,1-DMH and 1,2-DMH alkylated guanine at O^6 and N^7 position. In 1,1-DMH treated cellular DNA, 0.23% of the total counts were present in N^7-methylguanine and 0.09% in O^6-methylguanine. In 1,2-DMH treated cells, 0.17% and 0.07% of total counts were present in N^7-methylguanine and O^6-methylguanine respectively. The ratio of O^6/N^7-methylguanine was nearly the same, 0.39. In both alkylhydrazine treated cellular DNA the greatest amount of label was in apurinic acid fraction (60-70%). Incorporation of one carbon fragment from both alkylhydrazines into de novo synthesized purines accounted for approximately 20-40% of radioactivity incorporated into DNA. This is comparable to that reported by Harris et al. (13) and Autrup et al., (2-4).

The pattern of alkylation produced in synchronized cells treated with a transforming dose of 1,1-DMH and 1,2-DMH (0.5 mM) was slightly different while the percentage incorporation of radiolabel into N^7-methylguanine was similar. The radiolabel localized in O^6-methylguanine of 1,2-DMH treated cells was only 12% of that observed in 1,1-DMH treated DNA. Approximately 90-91% of total radiolabel was associated with the apurinic acid fraction and 4-8% was present in purine nucleus of adenine and guanine. In animal systems it is known that 1,2-DMH requires metabolic conversion into the ultimate carcinogen. Recent studies have identified azomethane, azoxymethane and methylazoxymethanol as metabolites of 1,2-DMH (47). Assuming that similar pathways may be operative in human cells, we

studied the alkylation of HNF cellular DNA by MAMA. Here again O^6-methylguanine and N^7-methylguanine represented 0.08 and 0.45% of total counts in DNA. Major incorporation of the label was localized in the apurinic fraction (84%) followed by unmethylated purines (15%). It was interesting to note that although 1,2-DMH was metabolized more extensively than either 1,1-DMH or MAMA (as judged by its binding to DNA), it was a poor transforming agent.

In human cell systems, mutagenicity by alkylating agents formation and persistence of 7-methylguanine and O^6-methylguanine, and their repair, has been reported (26), but there is a paucity of information available on relationships between transformation and presence of O^6-methylguanine or 7-methylguanine in human cells. Cells treated with transforming dose of 1,1-DMH and MAMA resulted in DNA containing similar amounts of O^6-methylguanine, which also coincides with their transforming ability. However, presence of similar amounts of O^6-methylguanine in DNA of cells treated with a non-transforming dose of 1,1 and 1,2-DMH and a decrease in O^6 methylation of guanine in DNA of cells treated with transforming dose of 1,2-DMH together with a fast repair of their lesion in human fibroblasts (26) suggest that factors other than O^6-methylguanine may play a role in transformation.

Sherer et al. (38) and Singer et al. (40) have shown that alkylated pyrimidine remains in the whole liver DNA longer than O^6-methylguanine. This in conjunction with observations of Abbot and Suffhill (1) and Singer (41) that O^4-methylthymidine and 3-methylcytosine can mispair suggest that O-alkylated pyrimidine may play a significant role in carcinogenesis.

A positive correlation has been reported between the ability of carcinogens to induce single strand breaks and their carcinogenic potencies (5). It is interesting to note that 1,1-DMH and 1,2-DMH which could transform HNF cells did not induce

any detectable DNA strand breaks, whereas MAMA, a potent transformer, did induce a small number of breaks ($0.27/10^8$ daltons) at a transforming dose. Low level of breaks seen with MAMA and absence of detectable breaks in DMH treated DNA suggest that strand break may not be a major factor for transformation at least in alkylhydrazine induced transformation of human cells.

It is known that any process interfering with endogenous methylation of cytosine at position 5 in the DNA may lead to a perturbed program of gene expression (7). DNA isolated from primary hepatocarcinomas derived from treatment of rat with three different carcinogens (N-2 fluorenylacetamide, chlordane and 3-methyl-4'-(dimethylamino)azobenzene) contains less 5-methylcytosine than does the DNA from normal liver tissue (20). DNA methylation in primary human tumor tissue, growth hormone and globin genes of cancer cells were found to be hypomethylated when compared with growth hormone and globin genes of normal cells from the same patient (11).

It is possible that 1,1-DMH, 1,2-DMH and MAMA can cause an abberent endogenous methylation of cytosine which can be stably inherited in the cell cycle that follows. A carcinogen induced damage can be fixed and an altered methylation pattern or specific deamination of methylcytosine would yield thymidine thereby creating a permanent miscoding of the genetic material. A single replication step is necessary and sufficient to fix these events and may also explain the role of cell proliferation in the initiation of chemical carcinogenesis in human cells.

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TABLE 1

TRANSFORMATION OF HUMAN FORESKIN FIBROBLASTS BY 1,1-DMH, 1,2-DMH AND MAMA

Ten hrs after release from block the cells were treated with the carcinogens for 12 hrs. At the end of the treatment period, the monolayers were rinsed 3X and split into a selection medium (see Carcinogen Treatment and Transformation, Materials and Methods). After 16-18 PDL, 50,000 cells were seeded into 2.0 ml of 0.35% agar in modified Dulbecco's LoCal medium over 5.0 ml of 2.0% agar base in RPMI 1629 growth medium. Three wks later plates were scored for colony formation. Each colony was counted that contained 50 or more cells. Untreated controls on occasion do form small colonies in soft agar. However, this procedure used here to evaluate anchorage independent growth is designed to discourage colony growth in soft agar, i.e. growth in LoCal Dulbecco's modified MEM + 20% FBS.

Carcinogen	Concentration (mM)	Number of Colonies Formed In Soft Agar per 10^4 Seeded Cells
1,1-DMH	0.03	—
	0.17	—
	0.50[a]	346 ± 69.8
	5.00 ($E.D._{25}$)	482 ± 63.0
1,2-DMH	0.03	—
	0.17	—
	0.23[a]	118 ± 36.9
	5.80 $E.D._{25}$	25.4 ± 10.9
MAMA	0.01[a]	50 ± 15.8
	0.03 ($E.D._{25}$)	340 ± 18.0

[a] These concentrations (mM) were non-toxic to the cells.

TABLE 2

METABOLISM OF 1,1-DMH, 1,2-DMH AND MAMA
BY HUMAN FORESKIN FIBROBLAST CELLS

Randomly proliferating cells in culture were treated with the carcinogen (0.17 mM, 12 mCi/mmole of 1,1-DMH or 1,2-DMH) for 24 hrs. Synchronized cells were treated 10 hrs after release from block with the carcinogen (0.5 mM, 1,1-DMH 15 mCi/mmole and 0.5 mM 1,2-DMH 7.2 mCi/mmole) for 6 hrs or with MAMA (0.027 mM, 44 mCi/mmole) for 6 hrs.

	Radioactivity Bound to DNA	
	dpm/mg DNA	pmoles/mgDNA
Randomly Proliferating Cells		
1,1-DMH	129,646	9,821
1,2-DMH	153,761	11,640
MAMA	N.D.	N.D.[a]
Synchronized Cells		
1,1-DMH	127,725	7,740
1,2-DMH	174,496	22,032
MAMA	114,980	2,380

[a] N.D. = not done

TABLE 3

METHYLATION OF DNA FROM RANDOMLY PROLIFERATING HUMAN FORESKIN FIBROBLAST CELLS TREATED WITH ALKYLHYDRAZINES

Cells were incubated with the carcinogen (0.17 mM, 12 mCi/mmole) for 24 hrs. Purified DNA was hydrolysed in 0.1 N HCl for 30 min. After the addition of markers, the liberated purines were separated on Sephadex G-10 column.

Fractions	1,1-DMH (0.17 mM)	1,2-DMH (0.17 mM)
Apurinic Acid	76.24 [a]	61.60
Guanine	9.20	16.50
Adenine	11.60	21.10
7-Methylguanine	0.23	0.17
O^6-Methylguanine	0.09	0.07
3-Methyladenine	0.64	0.50
O^6-Methylguanine/ N^7-Methylguanine	0.39	0.41

[a] Percentage of total adducts

TABLE 4

ALKYLATION OF DNA FROM SYNCHRONIZED HNF CELLS BY

1,1-DMH, 1,2-DMH and MAMA

Ten hrs after release from G_1 block cells were incubated with the carcinogen 1,1-DMH, 0.5 mM, 15 mCi/mmole; 1,2-DMH, 0.5 mM, 7.2 mCi/mmole; and MAMA, 0.027 mM, 44 mCi/mmole, for 6 hrs. Purified DNA was hydrolysed in 0.1N HCl for 30 min. Modified bases with the added markers were separated by HPLC.

Fractions	1,1-DMH (0.5 mM)	1,2-DMH (0.5 mM)	MAMA (0.03 mM)
Apurinic Acid	90.06	91.00	83.9
Guanine	2.30	5.60	11.0
Adenine	1.70	2.64	4.06
N^7-Methylguanine	0.24	0.20	0.45
O^6-Methylguanine	0.08	0.01	0.08
3-Methyladenine	0.26	N.D.	0.13
O^6-Methylguanine/ 7-Methylguanine	0.32	0.04	0.18

[a] Percentage of total adducts.

N.D. = not detected

TABLE 5

7-METHYLGUANINE AND O^6 METHYLGUANINE CONTENT IN DNA OF CELLS TREATED WITH MAMA

Ten hrs after release from block, the cells were treated with MAMA (S.A. 55 or 110 mCi/mmole). The purified DNA isolated from treated cells was hydrolysed in 0.1N HCl. Methylated purines were separated by HPLC on a cation exchange column as described under Materials and Methods.

Conc. of Carcinogen (mM)	Time of Treatment (hrs)	moles/10^6 moles of guanine		O^6MeG/N^7MeG
		7-MeG	O^6-MeG	
0.02	3	7.40	1.90	0.26
0.02	6	13.40	2.50	0.19
0.03	6	26.80	4.82	0.18

CHART LEGENDS

Chart 1. Cytotoxicity of 1,1-DMH, 1,2-DMH and MAMA. HNF cells (40 cells/cm^2 in 25 cm^2 dishes) were treated with the carcinogens for 24 hrs at different concentrations. After 7-10 days, cultures were fixed with 3% formalin for 20 min in PBS, stained with prefiltered hematoxylin and the colonies of more than 50-100 cells were counted under a 50X scope. These values (O, ●,Δ), represent $\bar{M}$ of n=8 and the vertical bar represents standard deviation from the M.

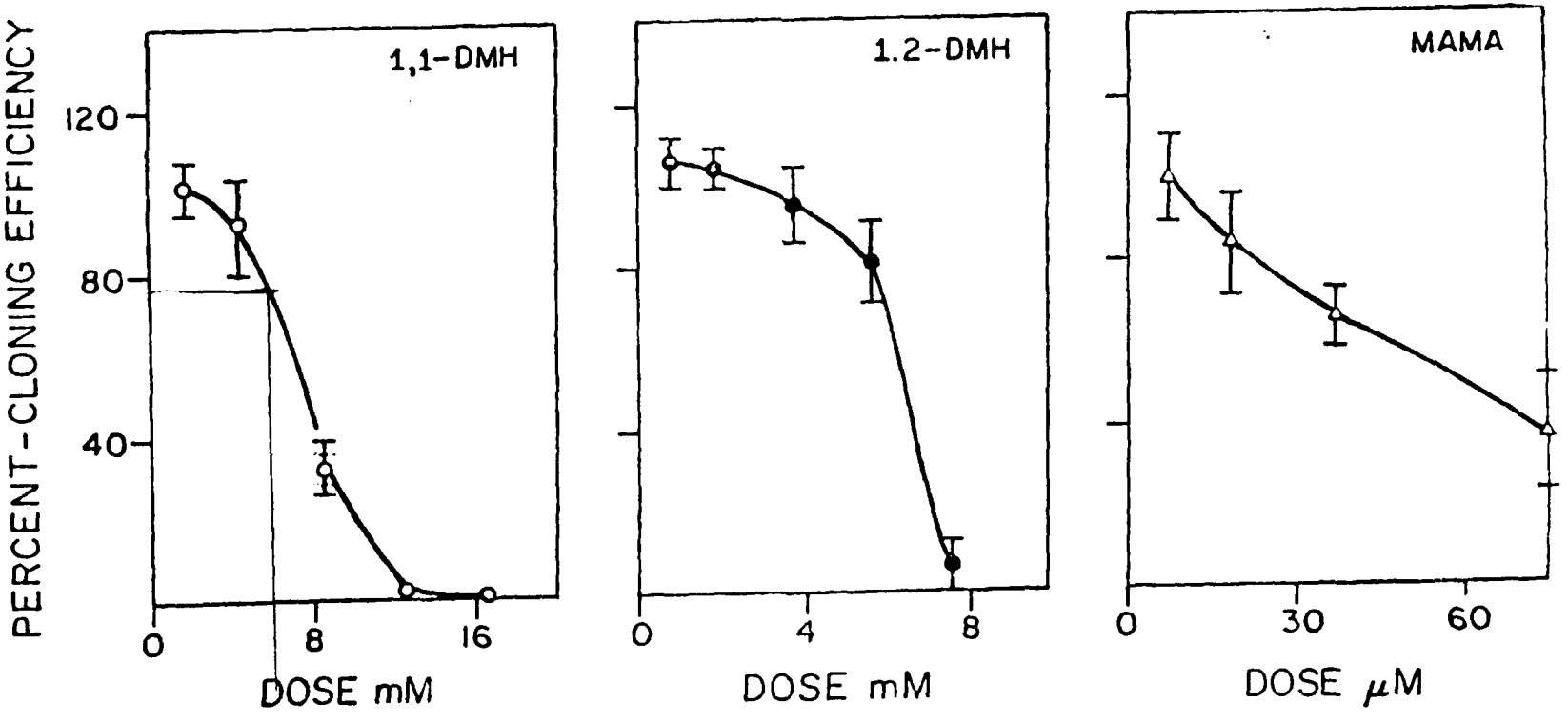


Chart 2. HPLC profile of acid hydrolysed DNA from 1,1-DMH treated cells (0.5 mM, 15 mCi/mmole). The sample was hydrolysed in 0.1N HCl at 70°C for 30 min. and chromatographed with standards on a strong cation exchange column. (Partisil 10SCX 9M in series with Partisil 10SCX). Methylated bases were eluted at 4 ml/min with a buffer which changed from 20 mM ammonoim formate in 6% methanol, pH 4.0, to 200 mM ammonium formate in 8% methanol, pH 4.0, over 25 min. using exp. 5 in LDC system. (——) represents radioactive profile. Gua, Guanine; N^2MeGua, N^2methylguanine; 7MeGua, 7-methylguanine; Ade, Adenine; O^6MeGua, O^6-methylguanine; 3-MeCyt, 3-methylcytosine; N^6MeAde, N^6-methyladenine; 7MeAde, 7-methyladenine; 1MeAde, 1-methyladenine; and 3MeAde, 3-methyladenine.

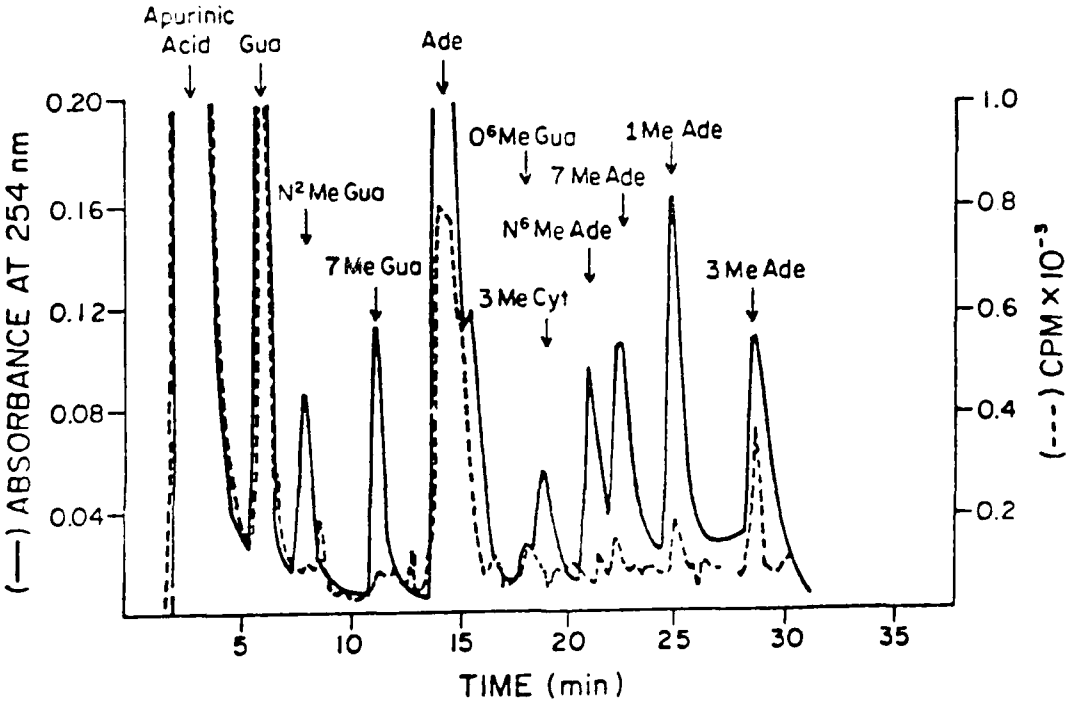
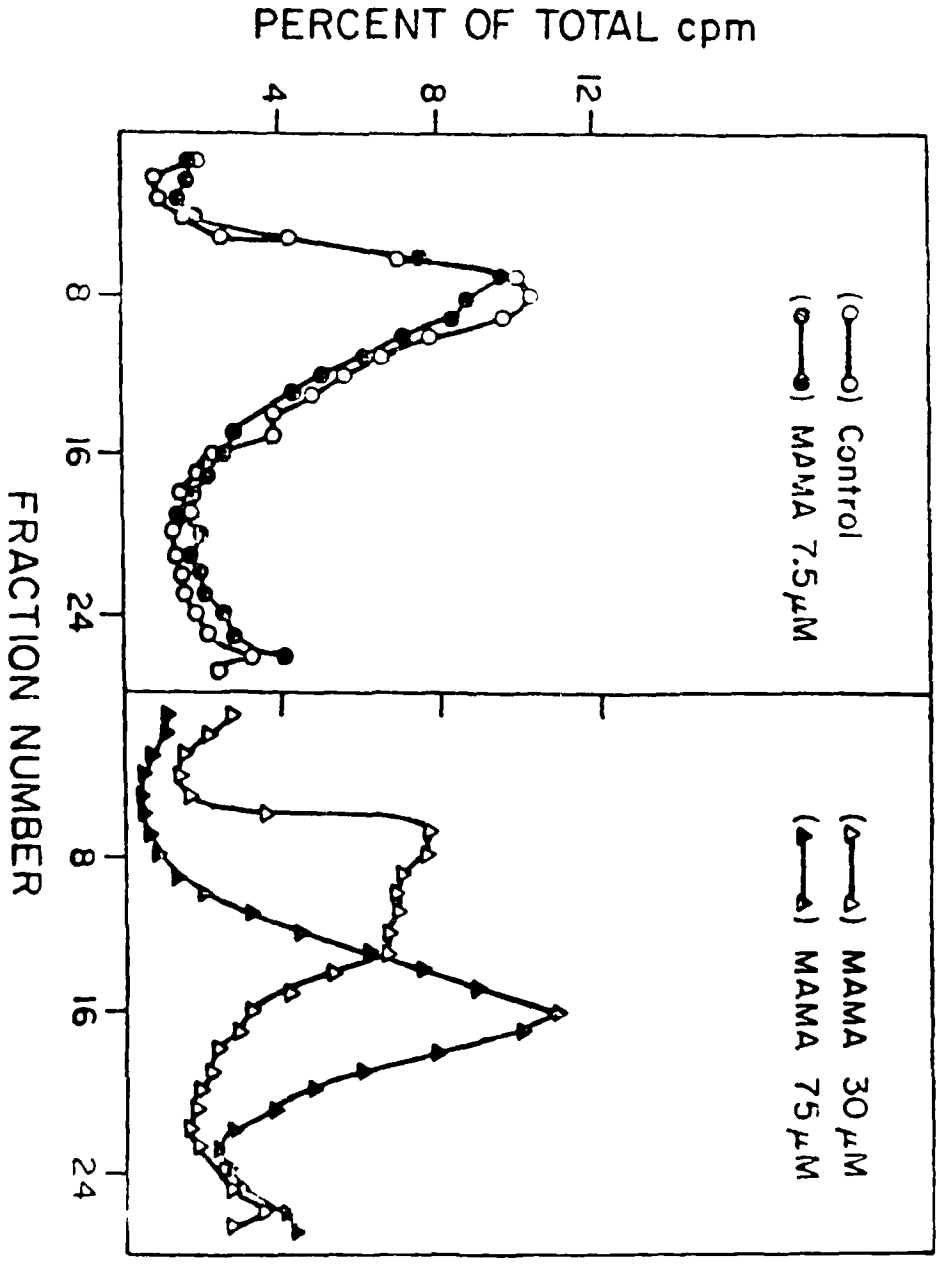


Chart 3. Sedimentation profile of DNA in alkaline sucrose gradient from synchronized HNF cells treated with MAMA for 12 hrs. Approximately 5 x 10^4 cells in 0.1 ml of PBS:EDTA were layered on top of 0.3 ml of lysis solution (1M NaOH: 10 mM EDTA) layered on top of 5-20% w/v sucrose gradient containing 2M NaCl, 0.33M NaOH and 0.01M EDTA and lysed in the dark for 3 hrs. The gradients were centrifuged in an S.W. 50.1 rotor for 145 min at 25K in a Beckman L8-55 ultracentrifuge. Approximately 25 fractions were collected and the radioactivity determined. (A) O—O control; ●—● MAMA 7.5 μM, (B)△—△MAMA 30 μM;▲—▲MAMA 75 μM.



Chapter V

Effect of the Anticarcinogen, Benzamide, on Molecular Peturbation of DNA by MAMA

INTRODUCTION

Methylazoxymethanol (MAM) which occurs as a glycoside cycasin in the seeds and roots of cycad plants, is a known potent carcinogen (1), mutagen (2) and a hepatotoxin (3). Studies in animal systems have demonstrated this compound to induce neoplasm in liver (4), lung (5), kidney (6) and brain (7) in various species including rats (8), mice (9) and fish (11). Since MAM is very unstable, methylazoxymethanol acetate is used in many studies. A number of reports have demonstrated that MAMA methylates the guanine residue of DNA and RNA at the N^7 and O^6 positions (12-14). Studies have demonstrated that MAMA inhibits rat liver nuclear RNA synthesis both in vivo (15) and in isolated nuclei (6). Fu-Liyu et al. (16) have also reported that MAMA is a potent inhibitor of nuclear and nucleolar RNA synthesis; it impairs chromatin template function and selectively inhibits RNA polymerase II activity. In addition it also causes condensation of nucleoplasmic chromatin. Rats treated with a low dose of MAM (20 μg/kg) exhibited no inhibition of humoral response (caused minimal damage to DNA in bone marrow, spleen and thymus), however, at a higher dose the effect on humoral immune response was non-uniform but did induce DNA damage.

While information is available on the carcinogenic potential and the biochemical effect of MAMA on animal systems, limited data has been reported on human cell systems except for that of Milo et al. (18) who have reported MAMA to be a more potent carcinogen than 1,2-dimethylhydrazine, a pro-carcinogen of MAMA (1,2-dimethylhydrazine on metabolism forms MAM in the animal system) (19).

This chapter describes the study on the effect of benzamide, an inhibitor of the poly (ADP-ribose) synthetase on MAMA induced a) transformation of human fibroblast cells, B) frequency of single strand breaks, and c) methylation of DNA bases.DNA damage induced by MAMA, its partial repair, (b) the methylation of cellular DNA, (c) effect of benzamide on single strand breaks, methylation of DNA and transformation caused by MAMA.

MATERIALS AND METHODS

Chemicals, Radiochemicals and Cell Culture Media

MAMA was obtained from Sigma Chemicals Co., (St. Louis, MO). The purity of the carcinogen was 98-99.5% as determined by thin layer chromatography and NMR. Radiochemicals, [2-^{14}C]-thymidine (S.A. 54.6 mCi/mmole) and [Methyl-^{3}H]-thymidine (S.A. 20 Ci/mmole) were purchased from New England Nuclear Corporation, (Boston, MA). [^{14}C]-MAMA (S.A. 110-112 mCi/mmole) was synthesized in the radiochemistry laboratory at The Ohio State University Comprehensive Cancer Center. Powdered cell culture media and supplements were obtained from the Grand Island Biological Company, (Grand Island, NY) and MA Bioproducts, (Walkersville, MD), respectively.

Cell Culture

Primary cultures of human neonatal foreskin fibroblasts (HNF) were obtained from routine circumcision tissue and the cells dispersed with collagenase and seeded in 75 cm^2 flasks. The primary fibroblast cultures were prepared by selective trypsin treatment (20). These cell populations at low population doublings (PDL) were serially subpassaged in complete medium (CM) composed of minimum essential medium (MEM), containing 25 mM Hepes at pH 7.2 supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2.0 mM glutamine, 50 μ g/ml gentocin, 0.2 % sodium bicarbonate and 10% fetal bovine serum in a 4% CO_2 enriched atmosphere.

Synchronization

Cells were synchronized by means of arginine and glutamine deprivation, (18,20,21). Briefly, cultures confluent for no more than 12-16 hrs were trypsinized with 0.1% trypsin and plated at desired concentration (0.5-1 x 10^4 cells/cm^2) in Dulbecco's

modified MEM lacking arginine and glutamine but supplemented with 10% dialysed fetal calf serum. Cultures were held in this medium for 24 hrs and released from this block by feeding with CM supplemented with 0.5 U/ml insulin. For transformation or alkylation of cellular DNA, ten hrs after release from the block when the cells were at G_1/S phase (as determined by ^{3}H-thymidine uptake and autoradiography) cultures were treated with the carcinogens for the specified time.

Transformation

Cells were seeded into T-25 flasks in arginine and glutamine free medium and synchronized as described above. In a typical experiment, cells were treated with the MAMA at 27 μM or with benzamide (1.0 mM) + MAMA (27 μM) for 12-14 hrs. Just before use, stock solution of MAMA was prepared in water and diluted in MEM. Benzamide was dissolved in ethanol. The control cells were treated with same concentration of ethanol. At the end of the treatment period, the cultures were rinsed 3X with CM minus serum, and split at 1:2 ratio into selection medium, i.e. CM containing 0.8 mM nonessential amino acids, 2X vitamins and 20% fetal bovine serum. When the culture reached 75% confluency, they were serially passaged at a 1:10 split ratio into selection medium. At 16-18 PDL (population doublings) 50,000 cells from rapidly proliferating non-confluent cultures were seeded into 2.0 ml of 0.35% agar in Dulbecco's modified LoCal medium supplemented with 1mM sodium pyruvate, 2 mM glutamine, 0.1 mM nonessential amino acids, 1X each of essential amino acids and vitamins, 50 μg/ml gentocin, and 20% fetal bovine serum over a 5.0 ml of 20% agar base made in RPMI 1629 growth medium (18,21) supplemented with 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM glutamine, 1X each of essential vitamins and essential amino acids, 50 μg/ml gentocin and 20% fetal bovine serum. The soft agar cultures were incubated at 37°C in a 4% CO_2 enriched air atmosphere. The cultures were fed once in seven days with approximately 0.2 ml Dulbecco's LoCal

medium. Three weeks later, the plates were scored for colony formation.

Treatment of Cells with Carcinogen and Isolation of DNA

At the onset of scheduled DNA sythesis, i.e., ten hrs after release from the block, radiolabeled [^{14}C-MAMA] (27 μM, S.A. 55 mCi/mmole) or 1 mM benzamide immediately followed by labeled MAMA was added to the cells two hrs into early S, and incubated for 3 hrs unless otherwise specified at 37°C. At the end of the treatment period the medium was removed from the treated cultures in 150 mm diameter plates, and the cells were rinsed 2X with MEM; the cells were scraped from the culture dish, and washed three times with MEM. The cell pellet was

finally suspended in SSC buffer (pH 7.0) at 10^7 cells/ml and lysed with 1% SDS. The cell lysate was extracted with an equal volume of redistilled phenol reagent (phenol 500 g, m-cresol 70 ml, 8-hydroxy quinoline 0.5 g, and 2X distilled water 55 ml), for 15 min. The contents were centrifuged at 10,000 x g for 30 min at room temperature to separate the organic-aqueous layers. The aqueous layer was removed and the phenol layer re-extracted with an equal volume of SSC buffer again. The pooled aqueous layers were re-extracted with phenol reagent. To the aqueous layer, 1/10 volume of 2M sodium acetate, at pH 5.0, was added followed by two volumes of ethanol at -19°C to precipitate the DNA. Any contaminating RNA and protein were removed from the DNA by incubating the DNA (1.0 mg/ml) in SSC buffer at 37°C for 30 min with 50 µg/ml of preheated pancreatic ribonuclease (80-100°C for 10 min) followed by incubation with pronase (predigested at 45°C for 1 hr in the dark), 100 µ g/ml for 60 min at 37°C. The DNA was reextracted again with an equal volume of phenol until there was very little interphase. DNA was finally precipitated with two volumes of 95% ethanol in the presence of 0.2 M sodium acetate. The precipitated DNA was washed with ethanol (-19°C) followed by ether (-19°C) and dried under a stream of oxygen-free nitrogen or argon.

The purified DNA was hydrolysed to purine bases and pyrimidine oligo nucleotides by heating at 70°C for 30 min in 0.1 M HCl and chromatographed with appropriate standards, by cation exchange high pressure liquid chromatography (HPLC). Chromatographic separation by HPLC was performed using a Laboratory Data Control (LDC) HPLC system with a Partisil-10 SCX M9 column (9.0 x 250 mm) in series with Partisil-10 SCX column (4.6 x 250 mm). The methylated products were eluted at 4.0 ml/min with a buffer which changed from 20 mM ammonium formate, pH 4.0, in 6% methanol, to 200 mM ammonium formate, pH 4.0, in 8% methanol over 25 min using a concave gradient.

Elution of purine and pyrimidine bases was monitored by absorbance at 254 nm. One ml fractions were collected and assayed for radioactivity. In calculating the alkylation of DNA, assumption is made that only one of the [^{14}C-]-CH_3 group is involved in alkylation.

Alkaline Sucrose Gradient Analysis

Randomly proliferating cells were labeled overnight with [methyl-^{3}H] or [2-^{14}C]-thymidine (1.0 or 0.2 μCi/ml respectively), incubated in fresh medium for 2 hrs and then treated with carcinogen for 12 hrs. At the end of the treatment period, cells were washed with PBS containing 0.04% EDTA and detached from the dish in 2.0 ml of PBS:EDTA using a rubber policeman. The cells were pelleted by centrifugation at 1000 rpm for 5 min in an IEC centrifuge, washed once and resuspended in PBS:EDTA at 5 x 10^5 cells/ml. The cell suspension containing approximately 5 x 10^4 cells was lysed in the dark for 3 hrs on a 0.3 ml pad of lysis solution (1 M NaOH: 10 mM EDTA) on top of a 4.5 ml, 5-20% w/v sucrose gradient containing 2 M NaCl, 0.33 M NaOH and 0.01 M EDTA according to methods described earlier (22). The gradients were centrifuged in an S.W. 50.1 rotor for 110 or 145 min at 25,000 rpm in a Beckman L8-55 ultracentrifuge. Approximately 25 fractions (0.2 ml each) were collected from the gradient and the radioactivity counted using 5.0 ml of Instagel scintillation cocktail in a Beckman LS 9000 scintillation counter. The gradients were precalibrated for molecular weights using the following phage DNA markers: lambda, T2 and T7. The frequency of alkaline labile lesions per 10^8 dalton of DNA was calculated using the formula: Alkaline labile lesions = 10^8 (1/Mnt - 1/Mno), where Mnt = Mn (the number average molecular weight) of DNA from carcinogen treated cells, and Mno = Mn of DNA from untreated cells.

RESULTS

Effect of Benzamide on MAMA Induced Transformation

As reported by Milo et al. (18) MAMA is a potent transformer. Exposure of cells to benzamide (1 mM) and MAMA (27 μM) at 10 hrs after release from G_1 block for 12 hrs, completely inhibited MAMA induced transformation. Addition of benzamide at G_1 release (MAMA is always added at 10 hrs after release) inhibited transformation by 33%. Addition of benzamide during the block (when added to synchrony medium for 24 hrs) or at late S phase resulted in no significant inhibition (Table I).

Alkaline Labile Lesions and Effect of Benzamide on Alkaline Labile Lesions

Fig. 1 illustrates the sedimentation profile of DNA from untreated control cells, cells treated with 75 μM MAMA for 3 hrs, and cells treated with 75 μM MAMA for 3 hrs and incubated for 4 hrs in carcinogen-free medium. As shown in Fig. 1, MAMA treatment induced single strand breaks and incubation of cells in MAMA free medium for 4 hrs resulted in partial repair of DNA as judged from its sedimentation at a higher molecular weight region. Fig. 2 shows that alkaline labile lesions increased as a function of dose and also time. However, a significant amount of damage is induced within one hr of incubation of cells with MAMA at 75 μM. Lack of significant increase in damage with time may be due to a rapid rate of repair. Addition of benzamide just prior to MAMA in early S had no effect on the extent of MAMA induced damage. (Table II). These results suggest that induction of DNA damage by MAMA may have no relevance to transformation. It may be more associated with mutation and cell cytotoxicity.

Alkylation of Cellular DNA by MAMA and Effect of Benzamide

Table III presents data on the extent of alkylation of DNA from cells treated with MAMA and MAMA plus benzamide. As shown in the table, similar amounts of

total alkylation of DNA was observed in the presence and absence of benzamide. After acid hydrolysis of this DNA in dilute acid to release the purine bases, the liberated purine bases were separated by HPLC. Both in MAMA and MAMA plus benzamide treated cellular DNA, approximately 92-93% of the label was associated with apurinic acid fractions. Radioactivity corresponding to 7-methylguanine (0.34-0.46%) and a very low detectable level of O^6 methylguanine was present (Table IV). There was also radioactivity in peaks which contained guanine (4-4.7%) and adenine (1.3-2.5%). This might have been due to denovo synthesis of purines from ^{14}C that might have entered the C-1 carbon pool. We consistently see modification of guanine at the O^6 position when the cells were treated with different transforming doses of MAMA. Table V presents data on the modification of guanine by MAMA in the presence and absence of benzamide. Again no significant difference was seen in 7-methylguanine content. Of the carcinogen-BZ treated cells compared to only carcinogen treated cells, a decrease in the O^6 methylguanine content was observed. If the inhibition we have observed is true then it suggests that O^6-methylguanine present in such a small amount plays a significant role in transformation.

DISCUSSION

Our work using HNF cells indicated that initiation of transformation by MAMA can be effectively inhibited when benzamide, at a concentration of 1.0 mM, is added to the cells at the early S phase, just before exposure to the carcinogen. However, benzamide at this concentration exhibited no cytotoxicity (results not shown).

Studies on DNA damage with MAMA and MAMA plus benzamide revealed that the addition of benzamide to the cells at a 1.0 mM level, just prior to MAMA had no effect on MAMA induced alkaline labile single strand breaks. This is in contrast to the results reported by other workers. Waters et al. (23) have shown that benzamide and 3-aminobenzamide at 5.0 mM concentration decreases the frequency of alkaline labile lesions in human fibroblast HSBP or IBR exposed to 3-methyl, 4-nitroquinoline. Morgan and Cleaver (24) have demonstrated that incubation of human cells, (primary human cells at passage 9 to 14) after exposure to the alkylating agents MMS, MNNG and MNU with 3-aminobenzamide (5 mM) reversibly enhanced the frequency of single strand breaks. However, the increase in single strand break frequencies was not observed in cells exposed to UV light. Thus these workers (25) propose that since the inhibitor had no effect on UV light and X-ray induced single-strand breaks, although transformation was inhibited, changes in DNA break frequency are unlikely to be involved in transformation. It is not clear why different groups are finding different effects. Is it because of different cell lines, different carcinogens, different regimen of treatment, or different method of assay used? Since we did not observe any enchancement or decrease in strand break frequency in our system it appears that strand breaks in DNA induced by MAMA has no correlation to MAMA induced transformation.

Absence of change in 7-methylguanine content observed in cells treated with a transforming dose of MAMA in the presence or absence of nontoxic dose of benzamide

suggests that 7-methylguanine may be involved in cytotoxicity rather than in transformation. We have further observed that modification of guanine at O^6 position was not appreciable altered by benzamide treatment. Recently, we have observed that carcinogen modification of the chromatin as measured by preferential binding studies (P. Kurian et al, manuscript under preparation) particularly in the core-linker of the nucleosome suggest very strongly that there is a conformational adjustment of chromatin in the presence of benzamide. To date we have found no significant difference in specific adduct formation in the presence and/or absence of the inhibitors.

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TABLE I

EFFECT OF BENZAMIDE ON MAMA-INDUCED TRANSFORMATION AS DETERMINED BY ANCHORAGE INDEPENDENT GROWTH IN SOFT AGAR

Treatment	Time of Treatment After Release from Block (hrs)	Length of Treatment (hrs)	# Colonies/ 10^5 Cells	% of Control
MAMA	10	14	750 ± 58	100
MAMA + Benzamide	0	10	234 ± 16	31
MAMA + Benzamide	-24	24	507 + 38	67
MAMA + Benzamide	10	14	1 ± 3	< 1

In all cases, cells were treated with MAMA (27 μM) at 10 hrs after release at G_1/S phase. Benzamide concentration was 1 mM. Number of colonies formed in soft agar is expressed per 10^5 cells. However, 50,000 cells were seeded per well in 8 dishes. Cells were treated with benzamide (1.0 mM) at the time point indicated in the table.

TABLE II

EFFECT OF BENZAMIDE ON ALKALI-LABILE LESION IN DNA INDUCED BY MAMA

Treatment	Alkali-Labile Lesions X 10^{-8}dalton
MAMA 30 μM	0.52 ± .17
MAMA 75 μM	1.16 ± .47
MAMA + 30 μM Benzamide 1 mM	0.6 ± .23
MAMA 75 μM Benzamide 1 mM	1.09 ± .36

Cells were treated with MAMA or MAMA + Benzamide for 12 hrs. DNA sedimented according to method described under Methods.

TABLE III

TOTAL BINDING OF MAMA TO CELLULAR DNA

IN THE PRESENCE AND ABSENCE OF BENZAMIDE

Treatment	pmoles/mg DNA
MAMA	409
MAMA + Benzamide	418

Ten hrs after release from the block, the cells were treated with MAMA (0.02 mM, S.A. 55 mCi/mmole) or MAMA + Benzamide (20 μM MAMA S.A. 55 mCi/mmole + 1.0 mM Benzamide) for 3 hrs.

TABLE IV

METHYLATION OF DNA OF HNF CELLS TREATED WITH MAMA OR MAMA + BENZAMIDE

Fractions	MAMA(%)	MAMA + BZ(%)
Apurinic Acid	93.6	92.3
Guanine	4.16	4.7
Adenine	1.32	2.5
7-Methylguanine	0.46	0.34
O^6-Methylguanine	0.12	0.04
3-Methyladenine	0.08	0.06

Percentage of total counts. For details see under Methods section.

TABLE V

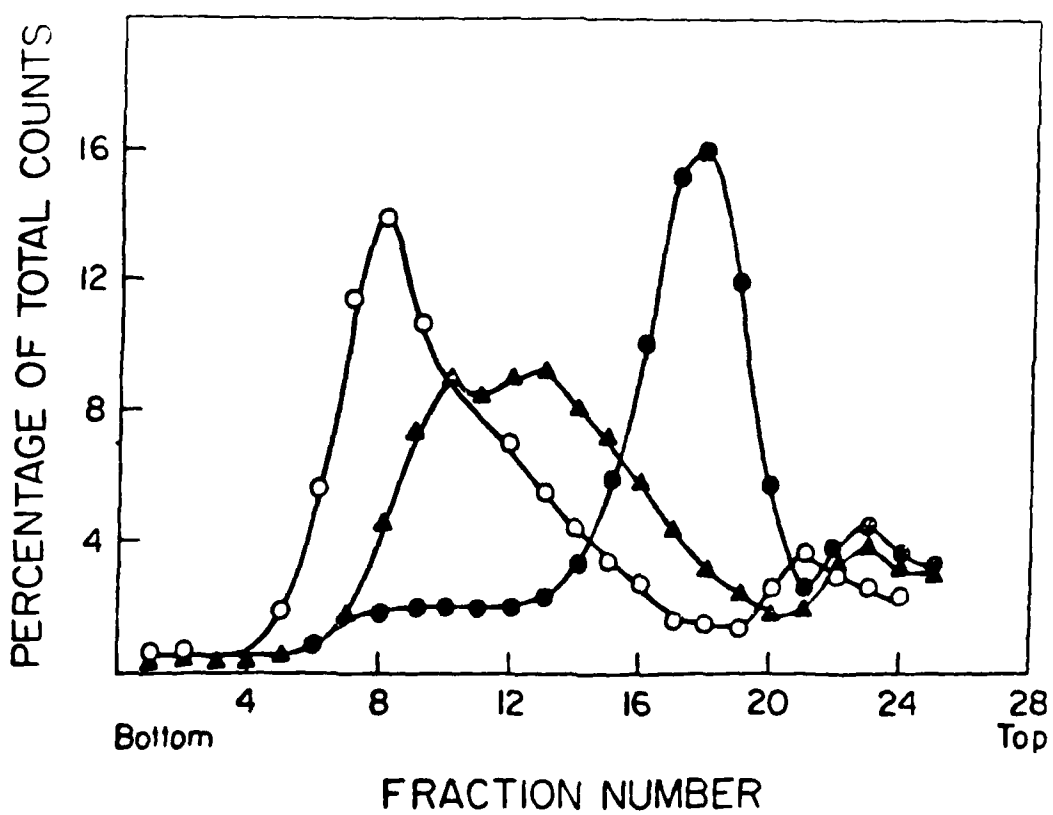
ALKYLATION OF DNA FOLLOWING TREATMENT WITH MAMA OR MAMA + BENZAMIDE

Treatment	μMoles Methylated Guanine/Mole Guanine	
	O^6Methylguanine	7-Methylguanine
MAMA	1.94	7.45
MAMA + BZ	1.1	8.52

For details see under methods. Benzamide (1.0 mM) was added to the cells just before the addition of MAMA (25 μM, S.A. 55 mCi/mmole).

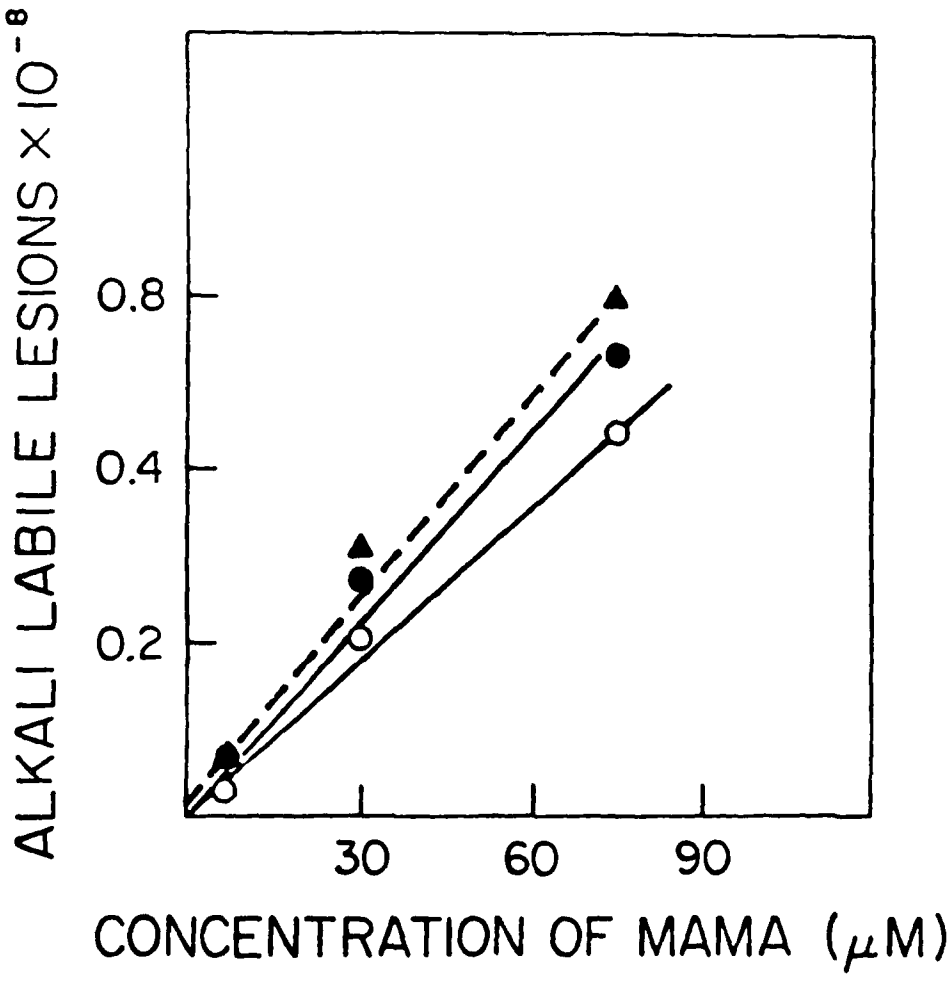
LEGENDS TO FIGURES

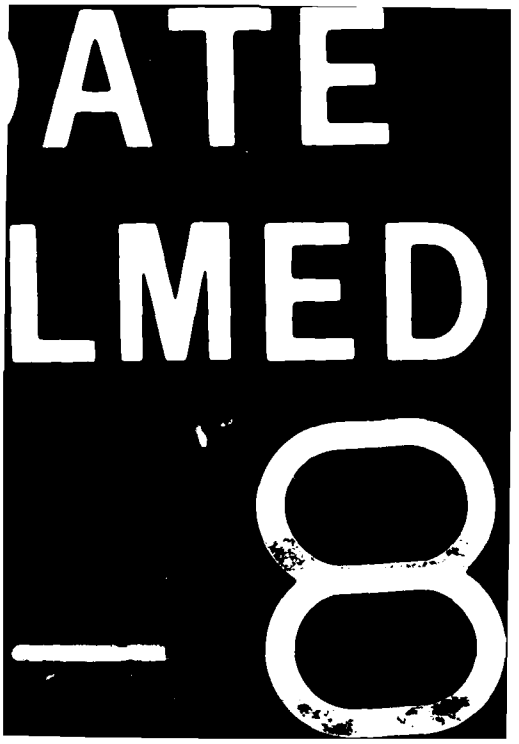
Fig. 1. DNA damage and repair. Sedimentation pattern of DNA from randomly proliferating cells treated with a) MAMA 75μM for 3 hrs.●——●, b) MAMA 75 μM for 3 hrs. followed by incubation in carcinogenfree medium for 4 hrs.▲——▲, and c) untreated cells O——O. Treated cells were prelabeled with ^{3}H-thymidine and were mixed with control cells prelabeled with [^{14}C]-thymidine. Centrifugation was done at 25,000 rpm for 145 min in a S.W. 50.1 rotor.



LEGENDS TO FIGURES

Fig. 2. Dose-response curve for alkaline-labile lesions produced in HNF cells by MAMA. Randomly proliferating prelabeled cells were treated with different concentrations of MAMA a) for 1 hr. O——O, b) 12 hrs. ●——●, c) 24 hrs. ▲- - - -▲. At the end, the treatment cells were processed according to the procedure described under Methods section. Centrifugation was done at 25,000 rpm for 110 min in a S.W. 50.1 rotor.

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